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Research Article

The Substitution Principle within the REACH Regulation: Nuclear Receptor-Bound Endocrine Disruptors

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Abstract. Within the REACH Regulation (EC/1907/2006), the substitution principle for chemicals classified as Substances of Very High Concern (SVHC) for either human health or environmental risks has been implemented in order to support their replacement *by suitable alternatives*. Considering the thousands of chemicals to be tested within the frame of REACH, animal testing by internationally-accepted guidelines sounds unreasonable in terms of the required time, costs as well ethical issues. Hence, REACH recommended also the use of alternative methods to animal experimentation although no validated *in silico* or *in vitro* tools were available when regulation entried into force. To search for suitable alternatives to SVHC having an Endocrine Disruptor (ED)-like Mode-of-Action (MoA) by means of an integrated, tiered *in silico-in vitro* approach, the EU-granted project LIFE-EDESIA (contract no. LIFE12 ENV/IT/000633) is combining computational-based tools and cell-based bioassays, in order to develop a no-animal testing procedure to screen for chemicals having less or no toxicity in terms of endocrine disruption-like activities. A general view of the no-animal testing approach implementing REACH and the substitution principle will be given, emphasising ligand-nuclear receptor (NR) assessment by molecular docking (one of the LIFE-EDESIA *in silico* approaches) and the use of clinical biomarkers in *in vitro* toxicology to detect ED-like adverse effects in cell-based bioassays.

Keywords: REACH regulation, Substances of Very High Concern (SVHC), substitution principle, Endocrine Disruptors, no-animal testing.

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1. Introduction

The *Registration, Evaluation, Authorisation and Restriction of Chemicals* (REACH, EC/1907/2006) Regulation [1, 2], entered into force on June 2007, is a *regulation of the European Union (EU), adopted to improve the protection of human health and the environment from the risks that can be posed by chemicals*.

In particular, the substitution principle [3] received a very important attention under the REACH process of Authorisation: chemicals posing high human health and/or environmental

risks and classified as Substances of Very High Concern (SVHC), not only have to be properly controlled but they have to be *progressively replaced by suitable alternatives*.

Chemicals identified as SVHC (REACH article 57) are those ones with the following hazard properties [1, 4]: i) carcinogenic, mutagenic or toxic for reproduction (CMR substances) [according to Commission Regulation EC/1272/2008)]; ii) persistent, bioaccumulative and toxic (PBT) or very persistent and very bioaccumulative (vPvB) [according to REACH Annex XIII]; and iii) existing scientific evidence, as demonstrated on a case-by-case basis, of probable serious effects that cause an equivalent level of concern as with CMR or PBT/vPvB substances (REACH article 57f). Among the latter chemicals are explicitly cited those ones fulfilling the criteria of *equivalent concern* as Endocrine Disrupting Chemicals (EDCs) or Endocrine Disruptors (EDs). Despite this, so far no internationally accepted criteria for identifying EDs exist, although many effort to set up and validate proper guidelines are ongoing.

As mentioned above, within the REACH Regulation there is a continuous reference to the substitution principle. It is clearly stated, indeed, that the eventual replacement of SVHC by suitable alternative substances have to be continuously pursued and that each authorization process should provide *an analysis of alternatives considering their risks and the technical and economic feasibility of substitution, including information on any research and development the applicant is undertaking or intends to undertake*. In other words, the substitution principle clearly indicate that when safer alternatives can be identified the extremely hazardous chemicals (*i.e.*, SVHC) should be replaced by such safer alternatives.

Lastly, REACH clearly supports also to the development of alternative tools to animal experimentation making a sharp reference to the Replacement, Reduction or Refinement of animal testing, the 3Rs principle [5], mentioning the objective of promoting no-animal testing and, above all, mentioning *in silico* and/or *in vitro* tools [6–8] as suitable to characterize certain dangerous properties of chemicals or even to elucidate a mechanistic understanding useful for the overall risk assessment of substances. Hence, the interest on alternative methods to animal experimentation ("no-animal testing"), namely computational (*in silico*) and cell-based (*in vitro*) methods [6–8] for toxicity testing, represent a priority issue within REACH focusing on the processes of screening and prioritization of chemicals but also exploiting their potential in hazard characterization. Indeed, another main goal of REACH is to promote *alternative methods for the hazard assessment of substances in order to reduce the number of tests on animals*.

To search for suitable alternatives to SVHC having an Endocrine Disruptor (ED)-like Mode-of-Action (MoA; REACH article 57f) by means of an integrated, tiered *in silico-in vitro* approach, the EU-granted project "Endocrine Disruptors *in silico/in vitro*—Evaluation and Substitution for Industrial Application" (LIFE-EDESIA, contract no. LIFE12 ENV/IT/000633, `http://www.iss.it/life`) is combining computational-based tools and cell-based bioassays, in order to develop a no-animal testing procedure to screen for chemicals that, in terms of endocrine disruption-like activities, could be considered safer and suitable for substitution in industrial applications.

Hence, the focus of the review will be the use of no-animal testing within the frames of the substitution principle and the REACH Regulation, highlighting the ligand-nuclear receptor (NR) assessment by molecular docking (one of the LIFE-EDESIA *in silico*, computational approaches) as well as the use of clinical biomarkers as biomarkers of effect *in vitro* toxicology to detect ED-like adverse effects in cell-based bioassays.

2. Endocrine Disruptors as Substances of Very High Concern and the Concept of Adversity

As mentioned within the Introduction, existing European Community regulations in the field of chemicals, recommends the use of alternative methods (*in silico* and/or *in vitro*) to animal experimentation as a priority approach for the screening of those chemicals that can be considered as Substances of Very High Concern (SVHC). The European Chemicals Agency (ECHA) list of SVHC, namely those ones included in the REACH Annex XIV, should include also substances (*e.g.*, EDs) that can cause adverse effects in an endocrine-like MoA. To date, ED effects are recognized as mainly mediated by their interaction(s) with the same molecular mediators of hormones, namely the ligand-bound transcription factors known as Nuclear Receptors (NRs) [9].

A world-wide accepted definition of EDs, established in 2002 by the World Health Organization (WHO; `http://www.who.int/ipcs/publications/new_issues/endocrine_disruptors/en/`), states that an ED is *a substance or an exogenous mixture that alters the function (s) of the endocrine system and consequently causes health adverse effects in an intact organism, or its progeny or (sub)populations*. Hence, the critical point in ED-related toxicology rely on the concept of adversity, above all using no-animal testing. In other words, which type of functional changes can be envisaged as an ED adverse effect?

As a matter of facts, from the mechanistic point of view, some EDs act as hormone agonist or antagonist via direct interaction with a NR (experimentally detectable by a nuclear receptor binding assay) and the subsequent activation (experimentally detectable by a gene transactivation o gene reporter assay) of NR-target genes [10]. Other EDs, can act modulating any protein (*e.g.*, enzymes, transporters, NR coregulators) controlling, in turn, either hormone synthesis or its conversion or its delivery to a target cell [11]. Furthermore, some EDs has been shown to act via epigenetic mechanisms that can be also transgenerally inherited [12, 13].

Such mechanism-based tools and body of proof, largely used by the scientific community and under validation at the EU level by the EURL ECVAM [14], have the inherent weakness to detect a molecular event and a subsequent intra-nuclear effect, but without to show if there is any consequence as a downstream functional response of the cell, in which the chemical exposure has been tested.

On the contrary, within the LIFE-EDESIA project, different cell-based bioassays are used to search for an ED-like MoA relying on the *in vitro* evaluation of cell-specific, hormone-regulated biomarkers of effect—already in use in clinical practice as indicators of either a disease or a patho-physiological alteration—in order to detect the cellular response upon chemical exposure [8].

3. Nuclear Receptors

In humans, NRs constitute a superfamily of 48 ligand-activated transcription factors able to regulate cognate gene networks involved in key physiological functions such as cell growth and differentiation, development, homeostasis, or metabolism [9]. NRs are modulated either by endobiotics (*i.e.* endogenous chemicals such as hormones and some vitamins) or xenobiotics

(*i.e.*, xenoestrogens such as plasticizers and pesticides). Hence, they allow to integrate different, multiple signals between central and peripheral organs orchestrating hormone-dependent signaling and also acting as xenosensors. NRs are located within the cell, shuttling among different intracellular compartments although the best of our knowledge is related to its nuclear localization [9, 15].

NRs have the same basic structure, divided in 5 functional domains: A and B domains at the amino terminus provide a binding site for transcriptional co-regulators. C and D (hinge) domains enable DNA binding at discrete sequences and are involved in protein nuclear localization and influence binding of the receptor to other transcription factors. E and F domains bind to additional co-regulators, often through an LXXLL motif in co-regulatory proteins. The E domain also contains a ligand-binding sequence that structurally differs in different receptors to confer specificity to ligands and antagonists. Overall, these domains contribute to the ligand-induced changes in gene expression occurring through the combined action of co-regulators and NRs bound at specific DNA sites [9, 15].

To schematically summarize ligand-activated NR genomic action, it is worthmentioning that some steroid receptors, such as the AR, are primarily located in the cytoplasm as inactive monomers, bound to heat shock proteins (HSPs). Others, such as the ERα, are located as monomers primarily in the nucleus, although a small percentage may also be bound to HSPs in the cytoplasm [15].

In the case of the AR, ligand binding to the cytoplasmic receptors triggers release from the HSPs, receptor dimerization, alterations in receptor conformation and nuclear localization. In the case of the ERα, the ligand binds to NRs to promote dimerization and changes in receptor conformation. In all cases, nuclear dimerized receptors then bind to specific steroid-response elements (SREs) and interact with various co-regulators to modulate gene transcription through either repression or activation.

4. The LIFE-EDESIA Project

The LIFE-EDESIA ("Endocrine Disruptors *in silico/in vitro*—Evaluation and Substutution for Industrial Applications"; grant LIFE12 ENV/IT/000633) project has been planned to apply the substitution principle to EDs of *equivalent concern* (`https://www.iss.it/life`). In particular, the project aims to demonstrate a new, robust and cost-effective *in silico/in vitro* approach to evaluate suitable chemicals for replacing EDs of *equivalent concern*, thus supporting the application of REACH legislative framework on the substitution principle environment. Furthermore, the project was planned to demonstrate that the *in silico/in vitro* selected alternatives were feasible to be used in industrial applications.

Accordingly, the following stepwise sequence of secondary objectives were planned to:

- identify potential substitutive chemicals [*i.e.*, di-2-(ethylexyl) phthalate (DEHP) as representative of phthalates, bisphenol A (BPA) as representative of bisphenols and methyl paraben as representative of parabens], both on the basis of the state of the art and using available *in silico* approaches to identify new potential candidates;
- perform a comparative assessment of the different potential substitutive chemicals using the *in silico* approach;

- synthesize the selected substitutive chemicals (if not commercially available);
- confirm the *in silico* results by a comparative assessment trhough *in vitro* methods;
- to create prototypes that use the substitutive chemicals, and to assess them for release of chemicals.

The LIFE-EDESIA search for new alternative molecules focussed on three classess of chemicals, phthalates, bisphenols and parabens, widely used in industry as plasticizers, food contact materials or food and cosmetic preservatives, and known or suspected to act as EDs. Such a search to phthalates and/or bisphenols and/or parabens substitutives is including chemicals newly designed or already known ones, on the basis on the different abilities to activate or to interact with NRs as judged by different *in silico* approaches such as the Quantitative Structure-Activity Relationship (QSAR) and the molecular docking tools [7, 16–18].

Since LIFE-EDESIA focussed mainly on the main recognized molecular targets of the above mentioned three classes of chemicals [8], and/or the mostly studied ligand-bound NRs, the Estrogen Receptors (ER)-α and ER-β the Androgen Receptor (AR; both the wild-type ARwt and a mutated form ARmut) and the Peroxisome Proliferator-Activated Receptor (PPAR)-γ have been considered to apply a virtual screning and docking/scoring approach to demonstrate it is possible to join *in silico* simulation with *in vitro* test to obey to EFSA (European Food Safety authority) and REACH recommendation.

The REACH Regulation suggests companies to substitute animal tests with alternative methods to animal experimentation including *in silico* and *in vitro* specific tests. These are undoubtedly good ideas but if the number of chemicals to be considered is a huge numer, the "cost-side" point of view of the problem could become a great investment. Unless *in silico* and *in vitro* approaches are part of an integrated strategy. For this reason, an approach termed as "funnel approach" (see Figure 1) could be an interesting and no expensive *in silico* way to start an alternative, animal-free screening passing through all steps of the 3Rs principle conjugating Replacement, Reduction and Refinement in all depicted steps.

5. No-animal Testing: *In Silico* Approaches

With the aim to model a potential endocrine disrupting activity, the interaction between chemicals under investigation (*i.e.*, phthalates, bisphenols, parabens) and some NRs (*i.e.*, ERα, ERβ, ARwt and ARmut) was evaluated by using the coupling of docking simulations and proper rescoring procedures. Specifically, the coupling of GOLD, as docking software, and HINT (Hydropathic INTeraction) [19], as rescoring function, was chosen on the basis of previous studies demonstrating the higher reliability of HINT in respect to other scoring functions and the efficacy as re-scoring function to predict ligands interaction with several protein targets [20–25], including estrogen [26, 27] and androgen receptors [28]. The HINT scores provide empirical and quantitative evaluation of protein-ligand interaction, as a sum of all single atomic contributions. Since they correlate with the free energy of binding, low or negative scores correlate with the thermodynamic disfavor of protein-ligand interaction.

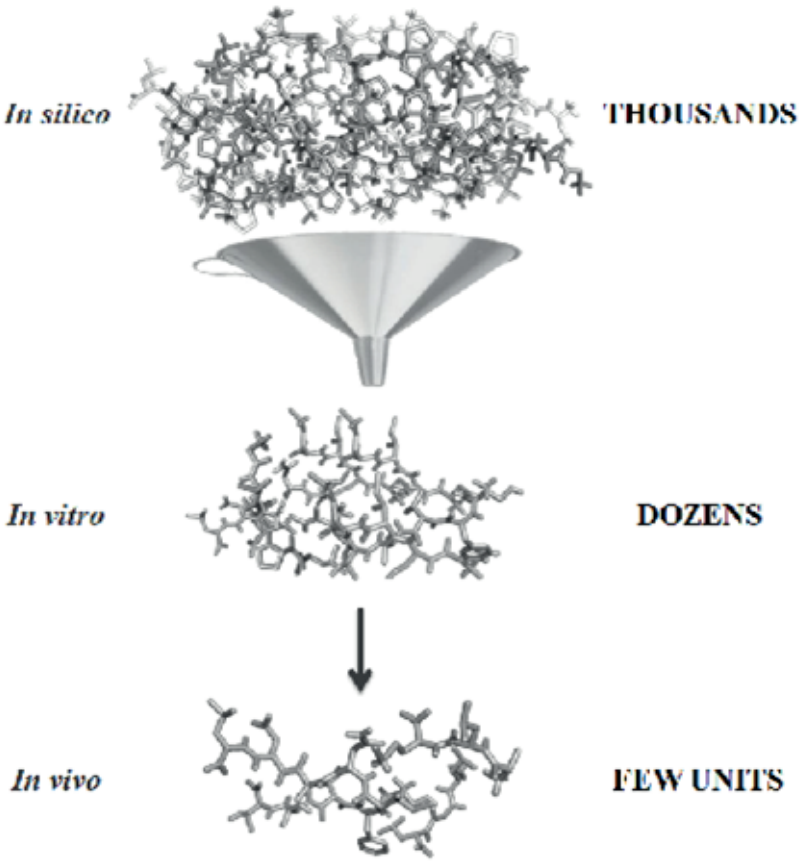

Figure 1: Schematic "funnel approach". An integrated toxicological screening of chemicals, from thousands (*in silico*) to dozens (*in vitro*) to few units (*in vivo*).

5.1. Molecular modeling

The model for ERα, ERβ, AR*wt* and AR*mut* derived from the Protein Data Bank (PDB) structures (`http://www.rcsb.org`) having PDB codes 2YJA, and 1X7C. All molecules and protein were processed using Sybyl 8.1 (`http://www.tripos.com`) to check atom and bond type assignments. Amino- and carboxy-terminal groups were set as protonated and deprotonated, respectively. Hydrogen atoms were computationally added and energy-minimized using the Powell algorithm whit a coverage gradient of ≤ 0.5 kcal (mol Å)$^{-1}$ and a maximum of 1500 cycles.

5.2. Docking simulations

Docking simulations of all compounds were performed with the program GOLD version 5.1 (CCDC; Cambridge, UK; `http://www.ccd.cam.ac.uk`) on a double-quad cores machine 1.86 GHz processors. All crystallographic waters and the co-crystalized ligand were removed and 50 poses for each compound were generated. No constraints were set up and the explorable space was defined in a radius of 10 Å from the centroid of the binding pocket. For each GOLD docking search, a maximum number of 100,000 operations were performed on a population of 100 individuals with a selection pressure of 1.1. Operator weights for crossover, mutation,

and migration were set to 95, 95, and 10, respectively. The number of islands was set to 5 and the niche to 2. The hydrogen bond distance was set to 2.5 Å and the vdW linear cut-off to 4.0. Ligand flexibility options "flip pyramidal N", "flip amide bonds", and "flip ring corners" were allowed. In order to consider the micro-flexibility of the pocket, the flexibility using the option "Library" for the following set of residues has been selected: Glu353, Phe404 and His524 for ERα; Glu305, Phe356 and His475 for ERβ; Thr877, Arg752, Gln711 and Asn70 for both AR*wt* and AR*mut*.

5.3. Rescoring procedure and hydrophatic analysis

The software HINT (Hydrophatic INTeraction)(REF) was used as post-processing tool. All poses generated by GOLD were re-scored in order to better evaluate protein-ligand recognition and, among the 50 poses computed for each compound, we carried forth only the highest scored pose. The HINT score provides empirical and quantitative evaluation of protein-ligand interaction, as a sum of all single atomic contributions, using the equation reported below:

$$\text{HINT score} = \sum_i \sum_j b_{ij} = \sum_i \sum_j \left(a_i\, S_i\, a_j\, S_j\, T_{ij}\, R_{ij} + r_{ij}\right)$$

where bij is the interaction score between atoms i and j, a is the hydrophobic atomic constant, S represents the solvent accessible surface area, Tij is a logic function assuming +1 or −1 values, depending on the nature of the interatomic interaction, Rij and rij are functions of the distance between atoms i and j. Positive and high HS correlates with favorable binding free energy, thus allowing evaluation of the thermodynamic benefit of predicted complexes [20, 22–24]. We termed this procedure "rescoring" because GOLD results are intrinsically ranked by its internal scoring functions. Because of this, it is not possible to separate GOLD's pose generation from its ranking, we were forced to apply HINT only to rescore ranked lists.

6. No-animal Testing: *In Vitro* Approaches and Functional Assays (or Biomarker-based Screening)

As mentioned above, in order to identify in an experimental *in vitro* system an ED-like adverse effect which, albeit indirectly, can also quantify an endocrine-like MoA, LIFE-EDESIA will take advantage of some experimental *in vitro* assays that are based on the measurement of cell-specific, hormone-regulated biomarkers of effect, using for this purpose clinical biomarkers as toxicological markers. The LIFE-EDESIA selected biomarkers are three different biomarkers of effect representative, at the same time, of three different cell-specific functionalities and three different endocrine-regulated targets that are under the control of the most known NR-signaling pathways (see Table 1).

The comparison of the modulation (either decreasing or increasing their secretion) of these biomarkers of effect between cells exposed to chemicals and cells not exposed (controls), can lead afterword to the identification and quantification of an ED-like adverse effect at the cellular level as summarized in Table 1. Indeed, the LIFE-EDESIA *in vitro* functional assays can be defined as "effect- and biomarker-based tools" or, in other words, as methods that based on the measurement of a cell-specific, endocrine-regulated biomarker of effect can inform about the adverse effects as caused by the chemical of interest [6, 8]. So, the LIFE-EDESIA selected cell

Table 1: **LIFE-EDESIA biomarkers of effects to detect an Endocrine Disruptor (ED)-like Mode-of-Action.** Human cell lines, human tissue targets and main nuclear receptor-dependent signalling pathways involved are also reported.

Cell-specific, hormone-regulated biomarkers of effect	Human cell lines	Endocrine Disruptor (ED) cell (or tissue) target	Nuclear receptor-, endocrine-dependent pathways controlling the selected biomarkers of effect
Prostate-Specific Antigen (PSA) secretion	LAPC-4 and LNCaP	Prostate epithelial-like cell (prostate gland)	Androgen Receptor (AR)-mediated signalling pathway
β human corio-gonadotropin (βhCG) secretion	BeWo	Trophoblast-like cells (undifferentiated, first trimester placenta)	Estrogen Receptors (ERs)-mediated signalling pathways
α-fetoprotein (AFP) secretion	HuH6	Fetal hepatocytes (fetal, immature liver)	Signalling pathways mediated by hormones regulating carbohydrate and lipid metabolism (*e.g.*, PPAR-α, -β -γ)

lines, of human origin, have to possess the feature to secrete, in an endocrine-regulated manner, the protein selected as biomarker because of the essential role for cellular activity.

As shown in Table 1, the secretion of the prostate-specific antigen (PSA), modulated by the androgen-regulated signaling pathway, is an indirect indicator of the functionality of the endocrine tissue of the prostate, the main male accessory gland, whose role is to secrete prostatic fluid, a third by volume of the male ejaculate, an essential component for male fertility. Hence, PSA measurement upon chemical exposure in the prostate epithelium-like cells, LAPC-4 and LNCaP, represent a proper functional, cell-specific, endocrine-regulated biomarker that, indirectly, provide information about an adverse effect—*e.g.*, altered semen quality via impaired protein secretion of a male accessory gland—connected to an androgen-like MoA [24, 25].

Similarly, the secretion of the human corio-gonadotropin beta (βhCG), modulated by the estrogen-regulated signaling pathways, is an indirect indicator of the proper cellular establishment, differentiation and maturation of the syncytio-trophoblast and subsequently of the placental tissue regulating the nutrient exchange between the mother-fetus dyad. Measurement of βhCG in chemically-exposed first trimester, trophoblast-like cells BeWo represent a proper functional, cell-specific, endocrine-regulated biomarker providing information about an adverse effect—*e.g.*, altered placentation via impaired cell differentiation—connected to an estrogen-like MoA [25, 26].

Finally, the α-fetoprotein secretion (AFP), modulated by a set of NRs including PPARs and RXRs, represent an indirect indicator of the proper accumulation of energy within immature, fetal hepatocytes as well as a correct lipid metabolism within the fetal liver and, as a consequence, its measurement in chemically-exposed fetal hepatocyte-like cells as HuH6 may represent a reliable, functional, cell-specific, endocrine-regulated biomarker useful to highlight an adverse effect—*e.g.*, altered fetal metabolism via impaired protein accumulation—connected to a PPAR-like MoA [27].

7. Conclusions

The use of an integrated *in silico-in vitro* approach in search for new and less hazardous chemicals has been proposed recently in different fields of research, including ED toxicology. In 2013, for example, it was defined a so-called Tiered Protocol for Endocrine Disruption (TiPED) [33], in which the proposed tiered approach was based on an *in silico-in vitro* screening to reduce the following *in vivo* experimental requirements. Indeed, the "funnel approach" depicted in Figure 1 has the same TiPED goal. Furthermore, as explained elsewhere [8, 16], the LIFE-EDESIA research strategy is also based on an *in silico-in vitro* tiered approach, whose aim is to take advantage of the *in silico* screening to select alternative chemicals in the above-mentioned "effect- and biomarker-based tools" in order to demonstrate reduced or absent ED-like adverse effects in different human cell lines representative of endocrine-targeted tissues. Hence, again similarly to the TiPED goal, but already defining *in vitro* the ED-like adverse effect [8], assessed by cell-specific, endocrine-regulated biomarkers of effect selected among the already used clinical biomarkers with the desired features to be applied as ED toxicological biomarkers (Table 1).

Finally, from the regulatory point of view, the scientific value of the above described new strategy of no-animal testing should be combined with a formal acceptance derived from a validation process that should be optimised for the specific issue of identifying EDs.

Competing Interests

The authors declare no competing interests.

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